Meaningful Detection of Known and Emerging Pathogens in Drinking Water

Project Scope

The provision of safe, treated drinking water requires the control of microbial contamination while minimizing health risks associated with the generation of disinfection by-products (DBPs). Essential to balancing these needs is the availability of microbiological monitoring methods that are practical, meaningful, and adaptable for both established and newly-discovered or emerging pathogens. Often, conventional methods are not sufficiently sensitive to detect small numbers of pathogens that may present risks to water consumers. Many common pathogens are difficult or impossible to cultivate in laboratory media, and polymerase chain reaction (PCR) detection of their genetic material in water, while very sensitive, is often of uncertain significance because of "false-positive" results due to the presence of dead cells or their components.

The overall goal of this research project was to develop and validate molecular methods for detecting viable bacteria in water using two nonstandard nucleic acid analytes. The first analyte, ribosomal RNA precursors (pre-rRNA), are intermediates in rRNA synthesis, which are abundant in growing bacterial cells but rare in nongrowing or nonviable cells. The second analyte, bromodeoxyuridine (BrdU)-labeled DNA, is a thymidine (a nucleoside component of DNA) analog that is incorporated into DNA during DNA replication, but which is not taken up by nonviable cells. Both approaches are species-specific, and both are diagnostic of viable cells capable of nucleic acid synthesis and growth under the test conditions. The researchers employed several bacterial model systems, including *Mycobacterium avium* (also known as *Mycobacterium avium intracellulare*, or MAC) and *Helicobacter pylori*, to test the feasibility of measuring bacterial pre-rRNA and BrdU-DNA in water supplies. The specific objectives of this research were to:

Grant Title and Principal Investigator

Meaningful Detection of Known and Emerging Pathogens in Drinking Water (EPA Grant #R826828)

Gerard A. Cangelosi, Seattle Biomedical Research Institute, Washington

Key Findings and Implications

Analytical Accomplishments:

- Developed a novel polymerase chain reaction-based assay using bromodeoxyuridine-labeled DNA that shows promise for detecting viable cells of *Helicobacter pylori* in drinking water.
- Discovered a new genetic marker (IS999) for *Mycobacterium avium* that may prove useful for tracking the evolution and spread of this important waterborne pathogen in the environment.
- Demonstrated that *M. avium* occurs in more morphologic forms than originally thought and that morphotype is related to virulence.
- Showed that the ability of *M. avium* to resist inactivation by disinfectant treatments such as chlorine occurs by mechanisms that are independent of the pathogen's intrinsic antibiotic resistance.

Implications of Research:

- Removing virulent forms of *M. avium* from drinking water may require less chlorine than is generally assumed, which could aid in the development of disinfection strategies that effectively protect against Mycobacteria while limiting disinfection by-product formation.
- Detection of *M. avium* colonies on standard growth media does not distinguish between virulent and chlorine-resistant forms; improved methods of *M. avium* detection (e.g., by growing colonies on Congo red agar) may yield better indications of virulence and the efficacy of drinking water treatment.

Publications include 5 peer reviewed articles, 1 book chapter, and 3 conference/workshop presentations.

Project Period: September 1998 to September 2001

Relevance to ORD's *Drinking Water Research Multi-Year Plan* (2003 Edition)

This project contributes directly to two of three Long-term Goals for drinking water research: (2) by 2010, develop new data, innovative tools and improved technologies to support decision making by the Office of Water on the Contaminant Candidate List (CCL) and other regulatory issues, and implementation of rules by states, local authorities and water utilities; and (3) By 2009, provide data, tools and technologies to support management decisions by the Office of Water, state, local authorities and utilities to protect source water and the quality of water in the distribution system.

This research project explored the feasibility of two novel technologies for detecting viable cells of two CCL pathogens, *Mycobacterium avium* and *Helicobacter pylori*, in drinking water. Although the researchers concluded that neither assay will likely prove useful for *M. avium* detection, the BrdU-labeled DNA method has some promise for detection of *H. pylori* in drinking water. The most significant results of this research were advances in knowledge concerning the biology of *M. avium* isolated from clinical and drinking water samples. Researchers demonstrated that *M. avium* occurs in more morphologic forms than originally thought, and that morphotype was related to virulence. They found that the most chlorine-resistant form of the pathogen, the "red-transparent" type, is not the most virulent form, and that resistance to chlorination and antibiotic exposure appeared to be mediated by different mechanisms.

1. Optimize pre-rRNA and BrdU-DNA analytes for use with PCR-based assays for the detection of viable bacteria in water;
2. Use pre-rRNA and BrdU-DNA procedures to study the growth physiology of *M. avium* in drinking water and its resistance to disinfection; and
3. Test the hypothesis that assays for pre-rRNA and/or BrdU-DNA can distinguish replicative (viable) from non-replicative forms of *H. pylori* in water.

Notably, because of their potential to be transmitted through drinking water, EPA placed *M. avium* and *H. pylori* on the first (1998) and most recent (2005) Contaminant Candidate List (CCL). The researchers conducted a series of experiments corresponding to each of the three objectives, the major findings and implications of which are summarized below.

Project Results and Implications

Optimization of Pre-rRNA and BrdU-DNA Assays: During the first year of this research project, quality assurance protocols for manufacturing novel reagents for use in the pre-rRNA and BrdU-DNA tests were developed using *Escherichia coli* and *Klebsiella pneumoniae*. Both assays performed well in these enteric microorganisms and were used in research conducted in support of objectives 2 and 3.

Studies of the Growth Physiology of *M. avium* in Drinking Water and Resistance to Disinfection: The pre-rRNA assay presumes that pre-rRNA pools are greatly diminished in nongrowing target cells. Although preliminary evidence for this approach using *E. coli* and *Mycobacterium bovis* (BCG; a bacterium closely related to *M. avium*) was promising, *M. avium* was found to maintain a large pre-rRNA pool even when growth was halted. This eliminated the pre-rRNA assay as an indicator of active growth and viability of *M. avium* in water and led to focused efforts on the development of the BrdU-DNA assay. As with the pre-rRNA assay, although the BrdU assay worked well in preliminary experiments using *E. coli*, *K. pneumoniae,* and BCG, *M. avium* cells did not incorporate significant amounts of BrdU or bromouracil (BrU; another thymidine analog) into their DNA regardless of the culture medium. The researchers concluded that *M. avium* appears to lack uptake mechanisms (e.g., active transport systems) for these compounds.

Because the pre-rRNA and BrdU-DNA assays failed to work well with *M. avium*, the researchers turned to traditional culture methods (i.e., plating and colony counting) to study the growth and survival of *M.*

avium in clinical and drinking water samples. Isolates of *M. avium* have been known to segregate into "colony types" (morphotypes) that vary that vary with regard to drug (e.g., antibiotic) susceptibility and virulence. Until recently, the reversible opaque-to-transparent colony type switch was considered the most important. Transparent variants predominate in clinical samples, are more resistant than opaque variants to most antimicrobials, survive well in water, and are generally considered to be the more chlorine-resistant than the opaque variants. This research led to the discovery of a new morphotype of *M. avium* (red-white) when opaque or transparent colonies were grown on agar media containing the lipoprotein stain Congo red. Most fresh clinical isolates of several strains of *M. avium* formed red and white colonies under these conditions with white opaque (WO) and white transparent (WT) variants shown to be more resistant to several antibiotics than red opaque (RO) and red transparent (RT) colonies. Within the transparent morphotype, red-white variation was consistently shown to affect virulence, with WT being consistently more virulent in both in vitro and in vivo studies. In addition, a preliminary examination of the morphotypic characteristics of *M. avium* cells isolated from several institutional water supplies in the Boston, Massachusetts area found the WT morphotype to be common, though additional research was planned.

Building on the finding that white variants of *M. avium* are more resistant than red variants to multiple antibiotics, the researchers next examined the susceptibility of both variants to chlorine. The goal was to test the common assumption that morphotypic water treatment disinfectant resistance and multi-drug resistance in *M. avium* are conferred by common mechanisms. The RT variant was found to be significantly more resistant to chlorine treatment than the three other forms, confirming intrinsic differences in chlorine resistance and suggesting that chlorine resistance and multi-drug resistance in red and white variants are conferred by independent mechanisms. Although these findings are based on observations of only two *M. avium* clinical isolates, and additional research using other strains was planned, the researchers suggested that chlorine-resistant forms of *M. avium* are not always the most virulent forms. This implies that removing virulent *M. avium* from drinking water may require less chlorine than is generally assumed, a finding that might be used to help develop effective disinfection strategies while limiting DBP formation. It also means that examination of *M. avium* colonies on standard growth media does not reliably predict virulence, and that the use of Congo red agar may yield better indications of disinfection efficacy.

During the course of characterizing the genetic basis for the red-white morphotype switch in *M. avium*, a novel mobile genetic element (IS999) was identified by the researchers. This insertion element is common in *M. avium* genomes but is less stable than IS1245, the most commonly-used marker for molecular epidemiological analysis of *M. avium*. The researchers concluded that IS999 will be a useful tool for tracking the rapid genetic drift that *M. avium* strains undergo in nature and under laboratory conditions.

Use of BrdU-DNA to Detect Viable *H. pylori*: *H. pylori* cells were grown with and without BrdU or BrU for 5 days under microaerophilic conditions. Although the BrU labeling did not provide consistent results, BrdU labeling was shown to work well at sufficient concentrations ($\geq$ 0.5 µg/ml). The researchers concluded that BrdU labeling method shows promise as a means of detecting viable *H. pylori* cells.

Investigators

G.A. Cangelosi, Seattle Biomedical Research Institute, Washington
J-P. Laurent, Seattle Biomedical Research Institute, Washington
S. Mukherjee, Seattle Biomedical Research Institute, Washington

For More Information
NCER Project Abstract and Reports:

http://cfpub.epa.gov/ncer_abstracts/index.cfm/fuseaction/display.abstractDetail/abstract/203/report/0

Peer Reviewed Publications

Cangelosi, G.A., Palermo, C.O., Laurent, J.-P., Hamlin, A.M., and Brabant, W.H. 1999. Colony morphotypes on Congo Red agar segregate along species and drug susceptibility lines in the *Mycobacterium avium-intracellulare* complex. Microbiology 145:1317-1324.

Cangelosi, G.A., Palermo, C.O., and Bermudez, L.E. 2001. Phenotypic consequences of red-white colony type variation in *Mycobacterium avium*. Microbiol*ogy* 147:527-533.

Mukherjee, S., Petrofsky, M., Yaraei, K., Bermudez, L.E., and Cangelosi, G.A. 2001. The white morphotype of *Mycobacterium avium-intracellulare* is common in infected humans and virulent in infection models. Journal of Infectious Diseases 184:1480-1484.

Laurent, J-P., Faske, S.M., and Cangelosi, G.A. 2002. Characterization of IS999, an unstable genetic element in *Mycobacterium avium*. Gene 294:249-257.

Laurent, J-P., Hauge, K.A., Burnside, K., and Cangelosi, G.A. 2003. Mutational analysis of cell wall biosynthesis in *Mycobacterium avium*. Journal of Bacteriology 185:5003-5006.